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Analysis of *ahpC* Gene Mutations in Isoniazid-Resistant Clinical Isolates of *Mycobacterium tuberculosis*

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The *ahpC* genes of 57 clinical isolates and one in vitro mutant of *Mycobacterium tuberculosis* were evaluated by nucleotide sequence analyses. Although compensatory *ahpC* promoter mutations were identified in 8 catalase-negative, *katG*-defective strains, the *ahpC* genes of 25 catalase-positive, isoniazid-resistant isolates and 25 drug-sensitive strains were not altered.

The mechanism of resistance to isoniazid (INH), the most effective and affordable agent for the treatment of tuberculosis, have not been defined in all *Mycobacterium tuberculosis* strains with reduced sensitivity to this drug. Mutations in the *katG* gene, encoding a catalase-peroxidase, or in the *inhA* locus, encoding a mycolic acid biosynthetic pathway enzyme, have been associated with resistance to INH in about 70% of *M. tuberculosis* strains (1, 3, 6, 7). Recent studies showing that overexpression of alkyl hydroperoxide reductase (AhpC) increases the MIC of INH for *M. smegmatis* have suggested that *ahpC* gene alterations may be a factor in INH resistance in the remaining *M. tuberculosis* strains (2, 14). Mutations in the *ahpC* promoter of *katG*-defective, catalase-negative, INH-resistant *M. tuberculosis* strains have been identified which enhance *ahpC* gene transcription and are apparently necessary to compensate for the loss of catalase-peroxidase activity (4, 10, 12). However, these mutations have not been shown to alter sensitivity to INH.

To further examine whether perturbations in the *ahpC* gene are associated with INH sensitivity in *M. tuberculosis* isolates, we examined strains with the following phenotypes: 8 catalase-negative, INH-resistant; 25 catalase-positive, INH-resistant; and 25 catalase-positive, INH-sensitive strains of *M. tuberculosis*. Fifty-seven of the strains analyzed were clinical isolates from Korea, Brazil, Mexico, China, and the United States, and the other strain (ATCC 35829) was an INH-resistant in vitro mutant of *M. tuberculosis* H37Rv. The INH MICs for and the catalase activities of these strains were determined as described previously (9). For all 58 strains, the 105-bp *oxyR-ahpC* intergenic region (containing the *ahpC* promoter) was amplified by PCR using *Taq* polymerase and primers designed from the published *ahpC* gene sequence (AHPC1 [5′-CCGCAACG TCGACTGGCTCA-3′] and AHPC2 [5′-ATTGATCGCCAA TGGTAAGC-3′]), cloned into the TA vector (Invitrogen, Carlsbad, Calif.), and analyzed for nucleotide sequence alterations with the *Bst*I Polymerase system (Bio-Rad, Richmond, Calif.) (11). To confirm the mutational analyses, at least two clones of each construct were sequenced. The 588-bp *ahpC* coding sequence of each of these isolates was evaluated by nucleotide sequencing after PCR amplification with primers AHPC3 (5′-ATGCATTGTCCGCTTTGATG-3′) and AHPC4 (5′-TTCTA TACTCATTGATT-3′). The *katG* and *inhA* loci of each strain were analyzed by PCR-single-stranded conformational polymorphism (SSCP) analysis, as described previously (6). Although various *katG* genotypes were identified, each strain had a wild-type *inhA* gene.

As seen in Table 1, all eight of the catalase-negative strains evaluated were highly resistant to INH, with MICs ranging from 10 to 125 μg/ml. Moreover, mutations that altered the KatG protein coding sequence were detected in all of these INH-resistant strains. Deletions were detected in three strains (K10, K23, and K29), a single-base-pair insertion was found in isolate K4, a nonsense mutation was detected in strain 35829, and missense mutations were identified in the other three isolates (K20, K28, and M8).

Nucleotide sequence analyses of the intergenic region upstream of the putative *ahpC* initiation codon demonstrated that all of the catalase-negative *M. tuberculosis* strains examined have alterations in *ahpC* regulatory sequences (Table 1). The promoter mutations were found at −6, −9, −10, −12, −30, and −42 (a novel T→C substitution) relative to the putative transcription initiation site (15). Previous promoter fusion, immunoblotting, and two-dimensional gel electrophoresis studies have shown that the same *ahpC* regulatory region mutations at −6, −9, and −12 result in upregulation of AhpC expression (10). The effects of the *ahpC* promoter mutations at −10, −30, and −42 on AhpC expression were investigated by subjecting cell extracts of K10 (C→T, −10), K28 (C→T, −30), and 35829 (T→C, −42) to immunoblot analysis with an antibody which recognizes mycobacterial AhpC (2, 12, 13). As seen in Fig. 1, K10 and K28 overexpressed AhpC relative to an *M. tuberculosis* H37Rv control. In contrast, AhpC overproduction was not detected in extracts prepared from 35829 (data not shown). This strain is among several *M. tuberculosis* isolates with *ahpC* promoter substitutions in which no detectable in-

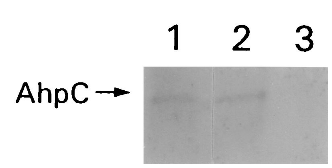

FIG. 1. Immunoblot analysis of extracts from *M. tuberculosis* clinical isolates with *ahpC* promoter mutations. Cellular extracts of isolates K10 (lane 1) and K28 (lane 2) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed for AhpC expression by immunoblotting using an antibody directed against *Corynebacterium diphtheriae* AhpC, which also recognizes mycobacterial AhpC. A cellular extract of the common laboratory strain H37Rv (lane 3) served as a control for this analysis.

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TABLE 1. *katG* and *ahpC* gene mutations in catalase-negative *M. tuberculosis* strains[a]

Isolate	INH MIC (μg/ml)	*katG* gene alteration (location)	*ahpC* promoter mutation
H37Rv	0.02		TATAT CACCTTTGCC TGACAGCGAC TTCACGGCAC GATGGAATGT
K4	125	Insertion (bp 1667)	.... A.........
K10	25	Deletion (bp 863–887)	....T ....
K20	50	H→Q (codon 108)	....T ....
K23	25	Deletion (bp 622–680)	....T.. ....
K28	50	H→Q (codon 108)	....T ....
K29	125	Deletion (bp 1–50)	....T ....
M8	10	F→L (codon 252)	....A......
35829	25	W→stop (codon 38)	..C.. ....

[a] Numbering of the *ahpC* promoter region nucleotides is based on the transcription start site, which is underlined and has been designated position 0. Nucleotides 5′ to the transcription initiation site have been given a negative numerical designation.

crease in AhpC levels have been demonstrated under the conditions assayed (12).

The second set of strains examined were 25 INH-resistant, catalase-positive clinical isolates. The INH MICs for these isolates were 1 to 2 μg/ml. Although *katG* perturbations were not detected in 15 catalase-positive, INH-resistant strains by PCR-SSCP analysis, the following missense mutations in *katG* were identified in the remaining 10 strains: 6 had R→L codon 463 mutations, 2 were altered at codon 315 (S→T), 1 was mutated at codon 334 (I→T), and 1 had a G→S codon 629 substitution. Nucleotide sequence analyses of these 25 isolates revealed no mutations in either the *ahpC* coding sequence or the *oxyR-ahpC* intergenic region. This result was not surprising for strains with wild-type *katG* genes or for isolates with codon 463 mutations. Recent studies have demonstrated that the codon 463 R→L alteration, the most common *katG* perturbation identified in INH-resistant *M. tuberculosis* strains, has little effect on enzymatic activity, and hence, *ahpC* compensatory mutations would not be expected (5, 8). Furthermore, the absence of *ahpC* mutations in the two codon 315 mutants examined in this study is consistent with previous observations on four other S→T codon 315 INH-resistant strains (12). Although the S→T substitution at this codon causes a 5- to 20-fold decrease in catalase-peroxidase activity, the residual enzymatic activity remaining in these mutants is apparently sufficient to allow in vivo survival in the absence of compensatory *ahpC* upregulation (3, 8). As controls, 25 INH-sensitive, catalase-positive clinical isolates were evaluated for *ahpC* gene alterations. No mutations were detected in the *ahpC* regulatory and coding sequences of these drug-sensitive strains.

In summary, our analysis of the *ahpC* regulatory and coding sequences of 58 *M. tuberculosis* isolates from geographically diverse locations supports the compensatory role of *ahpC* promoter mutations in *katG*-defective, catalase-negative tubercle bacilli. However, our failure to detect *ahpC* regulatory or coding sequence mutations in the catalase-positive, INH-resistant clinical isolates, especially those with wild-type *katG* and *inhA* gene loci, argues against the involvement of *ahpC* gene mutations in INH resistance. The mechanism(s) of INH resistance in *M. tuberculosis* isolates lacking alterations in *katG* or *inhA* remains unresolved and demands further careful investigation.

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